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Microglia Promote Learning-Dependent Synapse Formation through Brain-Derived Neurotrophic Factor

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SUMMARY

Microglia are the resident macrophages of the CNS, and their functions have been extensively studied in various brain pathologies. The physiological roles of microglia in brain plasticity and function, however, remain unclear. To address this question, we generated CX_3CR1^{CreER} mice expressing tamoxifen-inducible Cre recombinase that allow for specific manipulation of gene function in microglia. Using CX_3CR1^{CreER} to drive diphtheria toxin receptor expression in microglia, we found that microglia could be specifically depleted from the brain upon diphtheria toxin administration. Mice depleted of microglia showed deficits in multiple learning tasks and a significant reduction in motor-learning-dependent synapse formation. Furthermore, Cre-dependent removal of brain-derived neurotrophic factor (BDNF) from microglia largely recapitulated the effects of microglia depletion. Microglial BDNF increases neuronal tropomyosin-related kinase receptor B phosphorylation, a key mediator of synaptic plasticity. Together, our findings reveal that microglia serve important physiological functions in learning and memory by promoting learning-related synapse formation through BDNF signaling.

INTRODUCTION

Microglia are a population of resident myeloid cells that occupy all regions of the mammalian central nervous system (CNS). Recent studies have shown that microglia colonize the brain early during development (embryonic day 9.5) (Ginhoux et al., 2010). As development proceeds, microglia transition from an amoeboid to a highly ramified morphology with multiple fine processes that display a constant motility within neural tissues (Davalos et al., 2005; Swinnen et al., 2013). Although the role of microglia in CNS pathologies has been extensively studied, their contribution to normal CNS physiology remains unclear. Disruptions in the colony-stimulating factor 1 (*CSF-1*) signaling pathway in mice cause a reduction in the number of microglia, as well as defects in neuronal structure and function (Michaelson et al., 1996; Roumier et al., 2004). In humans, mutations in *CSF-1* signaling have been associated with presenile dementia (Paloneva et al., 2000). A variety of structural and functional deficiencies have also been associated with deletion or loss-of-function mutations in a number of genes expressed in microglia, including the fractalkine receptor CX_3CR1, methyl CpG binding protein 2 (*Mecp2*), and homeobox protein *HoxB8* (Chen et al., 2010; Derecki et al., 2012; Paolicelli et al., 2011). Together, these studies suggest that microglial dysfunction has a significant detrimental impact on the development and function of the CNS. However, because genes such as *CSF-1*, CX_3CR1, *Mecp2*, and *HoxB8* function in many myeloid populations, both microglia and peripheral myeloid cells were affected in these studies. Because deficits in peripheral myeloid cells could have significant effects on the CNS (Dantzer et al., 2008), caution is warranted in deducing the precise function of microglia in the brain from experiments using knockout mice that affect both peripheral and CNS myeloid cells.

Many lines of evidence indicate that experience-dependent synaptic structural plasticity is important for CNS development, as well as for learning and memory formation (Bailey and Kandel, 1993; Grutzendler et al., 2002; Yang et al., 2009a). For example, motor-skill learning induces the formation of postsynaptic dendritic spines in the motor cortex, and the survival of these spines strongly correlates with performance improvement after learning

(Liston et al., 2013; Yang et al., 2009a). Recent studies have shown that microglial processes are often in close proximity to neuronal somata and dendritic spines, and that the dynamics of microglial processes are regulated by sensory experience and/or neuronal activity (Tremblay et al., 2010; Wake et al., 2009). These findings suggest that microglia may play a role in regulating experience-dependent synaptic plasticity. Recently, several studies have suggested that microglia are involved in synaptic pruning through the phagocytosis of synapses during early postnatal periods, and that this process can be disrupted by loss of the fractalkine receptor *CX$_3$CR1* (Paolicelli et al., 2011) or complement receptor 3 (*CR3/CD11b*) (Schafer et al., 2012). However, synaptic phagocytosis by microglia and synaptic pruning defects observed in *CX$_3$CR1* and *CR3* null mice are absent during later postnatal development and adulthood (Paolicelli et al., 2011; Schafer et al., 2012). Furthermore, similar to other myeloid genes, *CX$_3$CR1* and *CR3* have functions not only in microglia but also in peripheral myeloid populations, which makes it difficult to pinpoint the precise functions of microglia using *CX$_3$CR1* and *CR3* knockout mice. Therefore, it remains unknown whether and how microglia are involved in experience-dependent changes of synaptic circuits, particularly in later postnatal and adult life. It is also unclear whether microglial dysfunction would contribute significantly to learning deficits as seen in neurological diseases.

To investigate the precise roles of microglia in the brain, we generated a mouse line that allows specific genetic manipulation of microglia in an inducible fashion. Here, we report that specific depletion of microglia leads to deficits in multiple learning tasks and learning-induced synaptic remodeling. Furthermore, genetic depletion of brain-derived neurotrophic factor (BDNF) from microglia recapitulates many of the phenotypes generated by deletion of microglia, indicating that microglial BDNF is an important factor for synaptic remodeling associated with learning and memory.

RESULTS

Generation of *CX$_3$CR1^{CreER}* Mice to Manipulate Gene Expression in Microglia

In order to manipulate microglial function, we generated *CX$_3$CR1^{CreER}* mice expressing tamoxifen-inducible Cre recombinase (CreER) in microglia under the control of the endogenous *CX$_3$CR1* promoter (Figure 1A). The gene encoding CreER was followed by an IRES-EYFP element and the insertion site was chosen according to previous studies in which the *CX$_3$CR1* coding region was replaced with enhanced GFP (EGFP) (Jung et al., 2000). Correct targeting of the *CX$_3$CR1* locus and subsequent flippase (FLP) recombinase-mediated excision of the neomycin resistance cassette was verified by Southern blot analysis (Figure 1B). We performed immunostaining for the microglial marker Iba1 and neuronal marker NeuN in brain slices from *CX$_3$CR1$^{CreER/+}$* heterozygous mice. As expected, we found that virtually all EYFP$^+$ cells (98.8% ± 1.3%, n = 172) were Iba1$^+$, whereas no NeuN$^+$ cells (n = 401) were EYFP$^+$ (Figure 1C), indicating that the CreER transcript is specifically expressed in microglia in the brain. To test the functionality of CreER, we crossed *CX$_3$CR1^{CreER}* mice to *Rosa26-stop-DsRed* reporter allele mice (*R26^{DsRed}*) (Luche et al., 2007) to generate *CX$_3$CR1$^{CreER/+}$:R26$^{DsRed/+}$* animals. In the absence of tamoxifen, few DsRed$^+$ microglia (0.3% ± 0.01%) were found in the brain of *CX$_3$CR1$^{CreER/+}$:R26$^{DsRed/+}$* mice (Figure 1D). In contrast, 5 days after tamoxifen treatment, the majority (93.9% ± 0.5%) of CX$_3$CR1-EYFP$^+$ microglia in *CX$_3$CR1$^{CreER/+}$:R26$^{DsRed/+}$* mice were found to coexpress DsRed (Figures 1D and 2A). Thus, Cre-mediated recombination in microglia is highly efficient in *CX$_3$CR1$^{CreER/+}$* mice.

Cre-Dependent Manipulation of Gene Expression in Microglia

Consistent with previous studies of *CX$_3$CR1* expression patterns (Jung et al., 2000), enhanced yellow fluorescent protein (EYFP) expression was also observed in multiple CD11b$^+$ myeloid populations in peripheral tissues of *CX$_3$CR1$^{CreER/+}$* mice (Figure 2A). We found that the percentages of several myeloid populations in the brain, blood, and spleen were comparable between wild-type (WT) mice and *CX$_3$CR1$^{CreER/+}$* mice at postnatal day 14 (P14) and P30, suggesting that the development and maturation of myeloid populations, including microglia in the brain, were not altered in heterozygous *CX$_3$CR1$^{CreER/+}$* mice lacking one copy of the endogenous *CX$_3$CR1* gene (Figures S1A and S1B available online). As expected, in *CX$_3$CR1$^{CreER/+}$:R26$^{DsRed/+}$* mice, Cre-mediated recombination occurred not only in CNS microglia but also in CX$_3$CR1$^+$ cell populations in the spleen (59.0% ± 3.1%), and blood (70.9% ± 4%) 5 days after tamoxifen administration (Figures 2A and 2C).

In order to restrict Cre-mediated recombination exclusively to microglia, we took advantage of the fact that microglia and other CX$_3$CR1$^+$ cells have substantially different rates of turnover and are derived from different precursor populations. Microglia are a self-renewing population (Ajami et al., 2007) with a low turnover rate (Lawson et al., 1992), whereas monocytes and inflammatory macrophages exhibit rapid turnover (van Furth and Cohn, 1968) and are replenished through a CX$_3$CR1$^-$ bone marrow precursor population (Fogg et al., 2006). Therefore, upon exposure to tamoxifen, we expect that microglia would undergo recombination that persists once tamoxifen has dissipated. Conversely, peripheral CX$_3$CR1$^+$ populations would initially undergo recombination but subsequently be replaced by nonrecombined cells from CX$_3$CR1$^-$ progenitors in the absence of additional tamoxifen (Figure S1C). Indeed, when *CX$_3$CR1$^{CreER/+}$:R26$^{DsRed/+}$* mice were pulsed with tamoxifen at P1–P3 and examined 30 days later, 93.0% ± 1.0% of EYFP$^+$ microglia were found to be DsRed$^+$ (Figures 2A–2C). In contrast, only 7.9% ± 1.9% of cells in the spleen and 1.7% ± 0.5% of the cells within the blood were DsRed$^+$ (Figures 2A and 2C). These results demonstrate that *CX$_3$CR1$^{CreER/+}$* mice, when utilized 30 days after tamoxifen administration, permit Cre-dependent manipulation of gene expression almost exclusively in microglia.

We next used a similar strategy to express the diphtheria toxin receptor (DTR) specifically in microglia, and administered diphtheria toxin (DT) to deplete microglia in the CNS while leaving other CX$_3$CR1$^+$ populations intact. To accomplish this, *CX$_3$CR1^{CreER}* mice were crossed with mice harboring the *Rosa26-stop-DTR* (*R26^{iDTR}*) allele (Buch et al., 2005), and *CX$_3$CR1$^{CreER/+}$:R26$^{iDTR/+}$* mice were given tamoxifen ~30 days

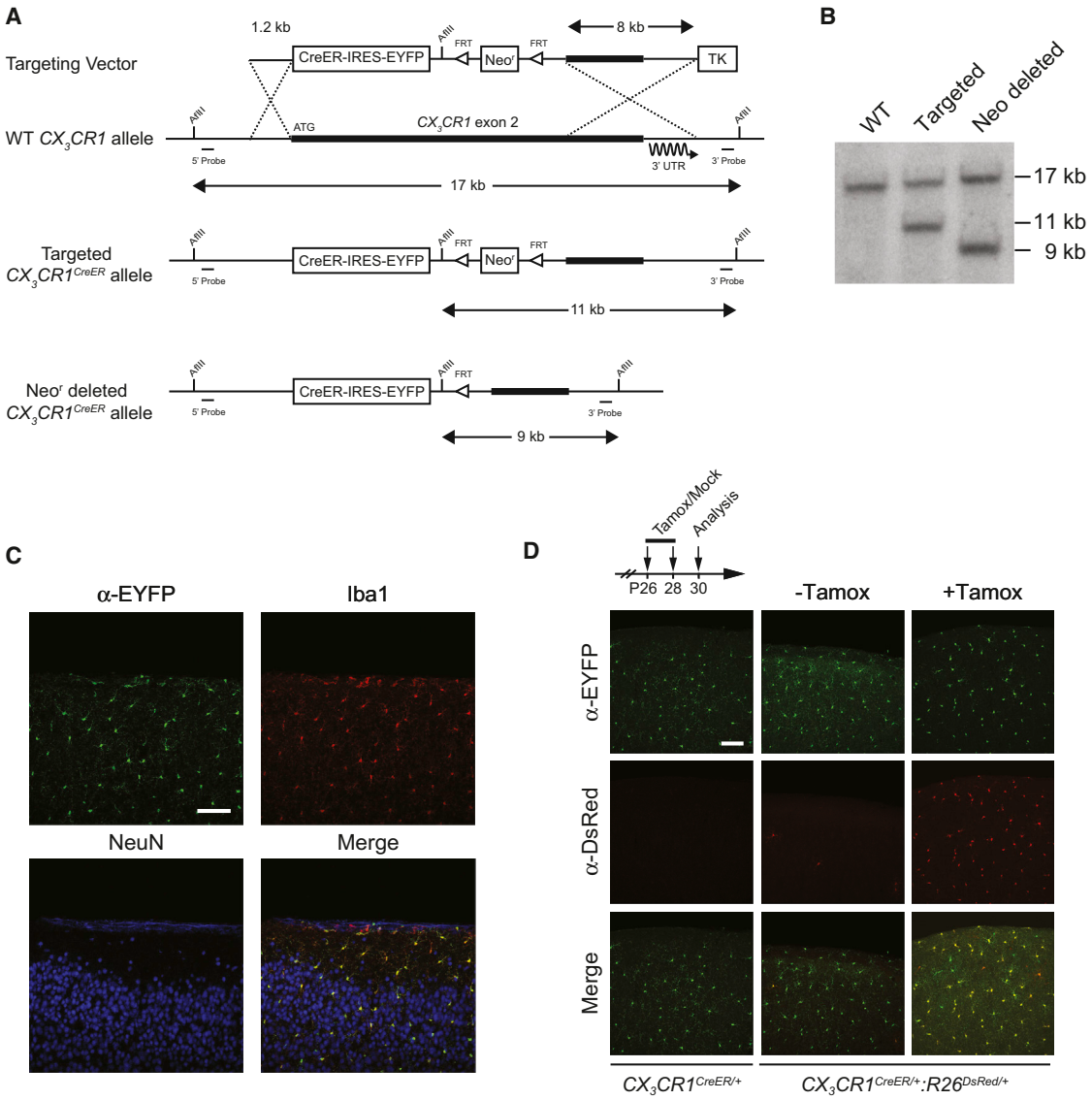

Figure 1. Generation of Mice Carrying the CX_3CR1^{CreER} Allele

(A) Schematic of the targeting strategy used for knockin of CreER-IRES-YFP at the *CX_3CR1* locus.

(B) Southern blot analysis of *AflII* digested genomic DNA from untargeted (WT), CX_3CR1^{CreER}-targeted, or CX_3CR1^{CreER} mice after deletion of the neomycin resistance sequence.

(C) Coronal sections of motor cortex from P45 CX_3CR1^{CreER} mice stained for EYFP, Iba1, and NeuN.

(D) Coronal sections of motor cortex from mice of the indicated genotypes and treatments (scale bar, 100 μm).

prior to the administration of DT (Figure 2D). We found that $CX_3CR1^{CreER/+}$:$R26^{iDTR/+}$ mice had a marked (99.1% ± 0.8%) reduction of CNS microglia within 1 day after administration of DT (Figures 2E–2G). In contrast, the number of microglia was not affected in the brain of control mice carrying a single copy of the CX_3CR1^{CreER} allele only. Seven days after DT administration, the number of microglia remained significantly lower (84.8% ± 3.0% reduction) in $CX_3CR1^{CreER/+}$:$R26^{iDTR/+}$ mice than in control mice (Figures 2E and 2G), suggesting limited repopulation of microglia within 1 week of depletion. Importantly, we observed no significant difference in the number of CX_3CR1^{+} $CD11b^{+}$ cells in the spleen or blood between $CX_3CR1^{CreER/+}$:$R26^{iDTR/+}$ mice and control mice (Figures 2H and 2I). These experiments demonstrate that $CX_3CR1^{CreER/+}$:$R26^{iDTR/+}$ mice can be used to specifically and robustly delete microglia in the CNS.

Within the first week after microglial depletion, we found no difference in animal viability (n = 14–16 per group) or weight change (Figure S2A) between microglia-depleted and nondepleted control mice. To investigate whether microglial ablation may cause inflammatory responses in the CNS, we measured transcript and protein levels of the inflammatory cytokines tumor necrosis

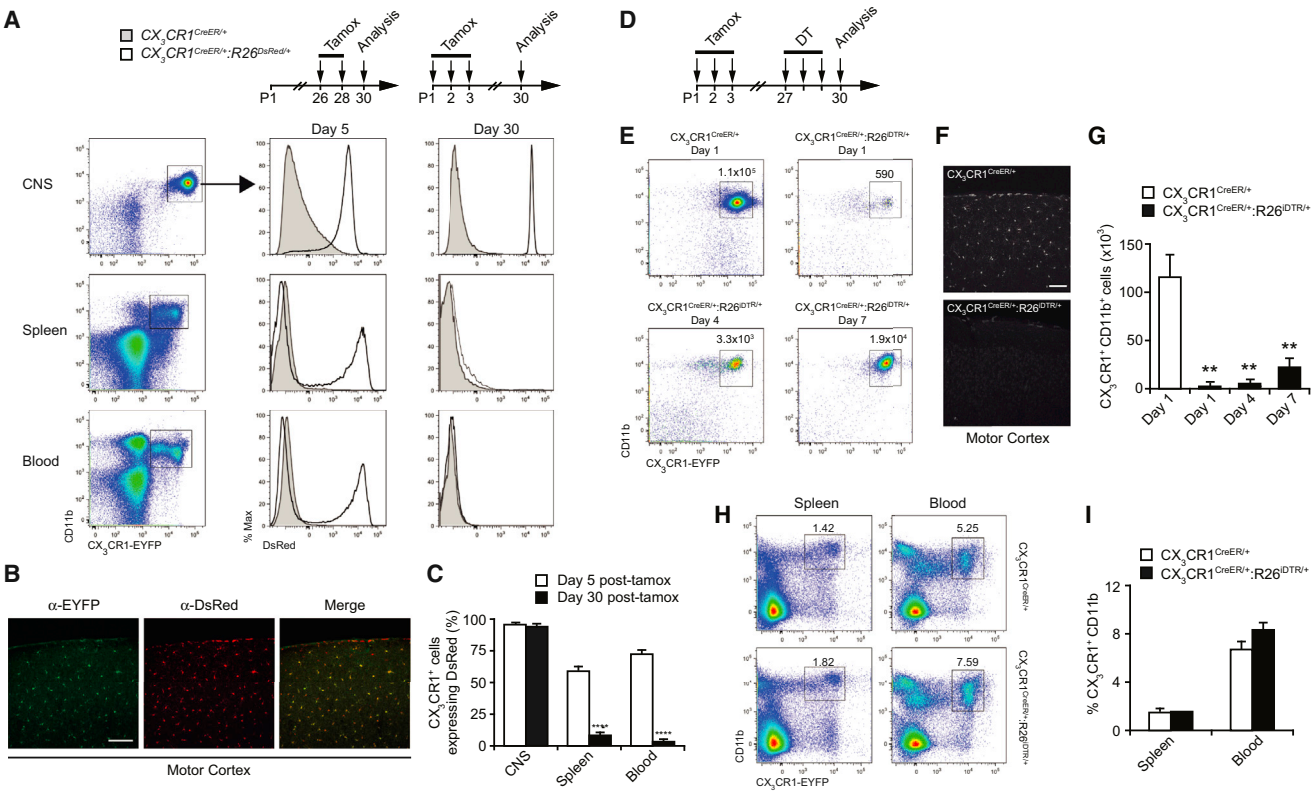

Figure 2. A Strategy to Restrict Cre-Mediated Manipulation of Gene Function, Including Deletion of Microglia

(A) CX_3CR1-EYFP$^+$ CD11b$^+$ populations in various tissues from mice of the indicated genotypes 5 or 30 days after tamoxifen treatment.

(B) Coronal sections of motor cortex from *$CX_3CR1^{CreER/+}$:$R26^{DsRed/+}$* mice stained for EYFP and DsRed 30 days after tamoxifen treatment.

(C) Quantification of flow-cytometry fluorescence-activated cell sorting (FACS) analysis showing the percentage of CX_3CR1-EYFP$^+$ cells coexpressing DsRed in multiple tissues at 5 or 30 days after tamoxifen treatment.

(D) Time course of tamoxifen/DT administration and analysis.

(E) FACS analysis of microglia in the brain of control and microglia-depleted mice at the indicated time points after DT administration. Dot plots show the total number of CX_3CR1-EYFP$^+$ CD11b$^+$ cells gated on DAPI$^-$ CD3$^-$ CD19$^-$ CD45^{int}.

(F) Coronal sections of motor cortex from control or microglia-depleted mice stained for Iba1 1 day after DT administration.

(G) Number of CX_3CR1-EYFP$^+$ CD11b$^+$ microglia in the brain after DT administration at various time points.

(H) FACS analysis showing the percentage of CX_3CR1-EYFP$^+$ CD11b$^+$ cells in the spleen and blood of mice after DT administration.

(I) Quantification of data shown in (H). n = 4 animals for each experimental condition. Data are represented as mean ± SEM. ****p < 0.0001, *p < 0.05. Scale bar, 100 μm.

See also Figures S1, S2, and S3.

factor α (TNFα), interleukin-1β (IL-1β), and IL-6 in brain tissues, and observed no significant difference between the microglia-depleted and control mice (Figures S2B–S2D). We also found no significant difference in the immunostaining of glial fibrillary acidic protein (GFAP) and glutamate transporter GLT-1 within 7 days after microglial depletion in either the motor cortex or hippocampal CA1 region (Figures S2E–S2J). In addition, we found no alterations in blood-brain-barrier permeability between microglia-depleted and control mice as measured by the amount of Evans Blue dye in the brain after intravenous injection (Figure S2K). To determine whether depletion of microglia altered the overall densities of neurons and synapses, we stained sections of the motor cortex and hippocampal CA1 region for NeuN, cleaved caspase 3 (CC3), or the presynaptic marker synaptic vesicle protein 2 (SV2) and found no significant difference in these parameters between microglia-depleted and control mice (Figure S3). Taken together, these findings suggest that depleting microglia leaves surrounding brain tissues minimally disturbed. Thus, *$CX_3CR1^{CreER/+}$:$R26^{iDTR}$* mice provide an important tool to delete microglia and examine their function in the CNS.

Depletion of Microglia Reduces Synaptic Structural Plasticity Associated with Learning

Recent studies have suggested that microglia participate in synapse elimination through a phagocytic engulfment mechanism involving the fractalkine receptor *CX_3CR1* (Paolicelli et al., 2011) or complement receptor 3 (*CR3/CD11b*) (Schafer et al., 2012). This process of synaptic phagocytosis seems to occur only during early, and not late, postnatal periods (Paolicelli et al., 2011; Schafer et al., 2012). To determine the potential function of microglia in the mature brain, we first investigated

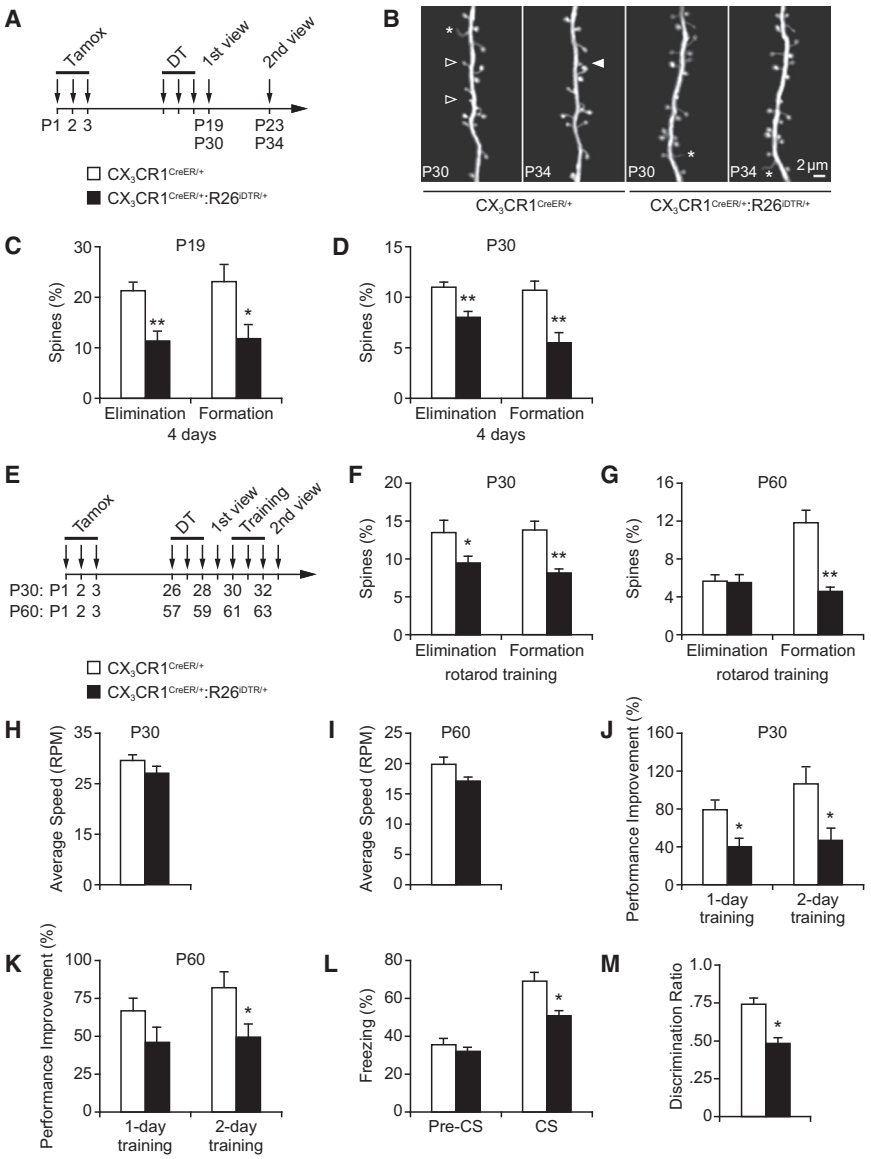

Figure 3. Microglia Are Important for Learning-Dependent Spine Remodeling and Performance Improvement

(A) Timeline of tamoxifen/DT administration and in vivo imaging in *CX_3CR1-iDTR* mice.

(B) Transcranial two-photon imaging of dendritic spines in control and microglia-depleted mice. Filled and empty arrowheads indicate spines formed or eliminated between two views. Asterisk indicates filopodia.

(C and D) The percentage of spines that were formed or eliminated within 4 days in the motor cortex was significantly reduced after microglia depletion in both P19 (C) and P30 animals (*$p < 0.05$, **$p < 0.01$, $n = 4–6$).

(E) Timeline of tamoxifen/DT administration, rotarod training, and in vivo imaging.

(F) Motor-learning-related spine remodeling was significantly reduced in P30 mice with microglia depletion (*$p < 0.05$, **$p < 0.01$, $n = 4–5$).

(G) Motor-learning-related spine formation was significantly reduced in P60 mice with microglia depletion (**$p < 0.01$, $n = 4–5$).

(H) Average speed reached during the first rotarod training session in P30 mice ($n = 6–7$).

(I) Average speed reached during the first rotarod training session in P60 mice ($n = 8$).

(J) Microglia-depleted mice showed impaired performance improvement compared with non-depleted control mice over 1 or 2 days of training (*$p < 0.05$, $n = 6–7$).

(K) P60 microglia-depleted mice showed impaired performance improvement compared with non-depleted control mice over 1 or 2 days of training (*$p < 0.05$, $n = 8$).

(L) Percentage of freezing in control or microglia-depleted mice before (pre-CS) and during (CS) presentation of the conditioned stimulus in the recall test (*$p < 0.05$, $n = 8$).

(M) The discrimination ratio of time spent interacting with a novel object versus a familiar object in a NOR assay was significantly altered in microglia-depleted mice (*$p < 0.05$, $n = 8$). Data are represented as mean ± SEM.

See also Figure S4.

whether microglia depletion might alter synaptic structural plasticity during the late postnatal period (P19) or young adulthood (P30). Using transcranial two-photon microscopy (Yang et al., 2009a), we examined the effect of microglial depletion on the baseline remodeling of postsynaptic dendritic spines of layer V pyramidal neurons in the motor cortex of Thy1 YFP-H line mice crossed with *$CX_3CR1^{CreER/+}$* or *$CX_3CR1^{CreER/+}$:$R26^{iDTR/+}$* mice (Figures 3A and 3B). We found that at either P19 or P30, microglial depletion caused a significant decrease in both spine formation and elimination over 4 days (Figures 3C and 3D). In addition, we observed that baseline spine remodeling was not altered in *$CX_3CR1^{CreER/+}$* mice lacking a single copy of the endogenous *CX_3CR1* gene, as *$CX_3CR1^{CreER/+}$:Thy1 YFP-H* and *$CX_3CR1^{+/+}$:Thy1 YFP-H* mice showed similar rates of spine turnover (Figure S4A). Taken together, these findings suggest that microglia are involved in not only spine elimination but also spine formation during late postnatal development and young adulthood.

Previous studies have shown that motor-skill learning causes an increase in dendritic spine remodeling in the motor cortex, and that the degree of new spine formation correlates with performance improvement after learning (Liston et al., 2013; Yang et al., 2009a). To investigate the role of microglia in this process, we examined whether depletion of microglia altered spine formation and elimination over 4 days in response to rotarod motor learning (Figure 3E). We found a significant decrease in learning-dependent formation and elimination of dendritic spines in 1-month-old microglia-depleted mice as compared with age-matched controls (Figure 3F). Furthermore, in 2-month-old adult mice, microglia depletion caused a significant decrease in learning-dependent spine formation, but not spine elimination (Figure 3G). Notably, although the baseline performance of

microglia-depleted animals remained unchanged (Figures 3H and 3I), there was a significant decrease in performance improvement after motor learning in microglia-depleted mice as compared with control mice at P30 and P60 (Figures 3J, 3K, and S4B). These results demonstrate that microglia have an important role in learning-induced remodeling of excitatory synapses, as well as in animals' performance improvement after motor learning.

To further understand the effect of microglia deletion on learning and memory, we compared microglia-depleted and control mice in two additional behavioral paradigms: auditory-cued fear conditioning (FC) and novel object recognition (NOR). We found that microglia-depleted animals exhibited significantly reduced freezing fear response to the auditory cue during the recall test as compared with nondepleted controls (Figure 3L). Additionally, whereas the nondepleted control mice showed a preference toward the novel object during the NOR test, the microglia-depleted mice showed no such preference (Figure 3M). Thus, mice lacking microglia demonstrate deficits in multiple behavioral tasks, underscoring an important role of microglia in learning and memory that involves multiple brain regions.

Depletion of Microglia Alters Synaptic Protein Levels and Glutamatergic Synaptic Function

Our results indicate that microglial depletion causes defects in learning-induced dendritic spine remodeling and behavioral performance. To better understand synaptic alterations after microglial deletion, we examined the levels of various proteins in microglia-depleted brain, focusing on those involved in synaptic plasticity and function. We first performed a quantitative proteomic screen from whole-brain protein extracts by shotgun multidimensional liquid chromatography–tandem mass spectrometry (LCLC-MS/MS) high-resolution mass spectrometric proteome analysis (Washburn et al., 2001). Specifically, we utilized ^{15}N-labeled mouse brains as an internal standard and mixed ^{15}N whole brain 1:1 with P30 microglia-depleted or control littermate brains to calculate relative protein profiles (Figure 4A). A similar approach was used previously for proteome-wide quantitative analysis of long-lived proteins and synaptosome changes during development (Savas et al., 2012). We quantified a total of 6,562 proteins and found that the levels of 61 proteins were significantly altered as a result of microglia depletion (Figure 4B; Table S1; ANOVA p value < 0.05, $\log_2$ fold change $\geq 20\%$). Importantly, 21/61 (34%) of the significantly altered proteins had known roles in synaptic plasticity/function, such as the postsynaptic glutamate (NMDA) receptor subunit epsilon-2 (GluN2B) and the presynaptic vesicular glutamate transporter 1 (VGlut1). We additionally found the glutamate (AMPA) receptor subunit 2 (GluA2) to be decreased, although it did not meet our significance criteria.

To further investigate the alteration of synaptic proteins after microglia depletion, we generated synaptosome fractions from microglia-depleted mice and compared them with those from the nondepleted controls. In line with the results of our proteomic screen, we found VGlut1 and GluA2 to be significantly decreased in synaptosomes from microglia-depleted brains. Although GluN2B protein was increased in the whole-brain fraction as shown in our proteomic screen, its level was decreased in the synaptosome fraction, suggesting differential alteration of GluN2B in synaptosome versus nonsynaptosome fractions after microglial depletion. The NMDAR subunit GluN2A and the AMPAR subunit GluA1 remained unaltered between microglia-depleted mice and controls (Figures 4C and 4D). These results support the data from our proteomic screen and demonstrate that microglia-depleted brains display significant and specific changes of synaptic proteins at glutamatergic synapses.

To test whether changes in the abundance of the synaptic proteins GluN2B, VGlut1, and GluA2 after microglia depletion affect glutamatergic synaptic function, we performed whole-cell patch-clamp recordings from layer V pyramidal neurons in the motor cortex of P30 mice 1 day after the depletion of microglia. We found that the frequencies of both NMDA and AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) were significantly decreased as compared with controls (Figures 4E–4H), suggesting a decrease in spontaneous glutamate release in the absence of microglia. Furthermore, the decay time of NMDA mEPSCs, but not AMPA mEPSCs, was significantly reduced in microglia-depleted animals, whereas the amplitude of spontaneous NMDA and AMPA mEPSCs remained unchanged (Figures 4F and 4H). The decrease in the decay time of NMDA mEPSCs is consistent with the reduction of GluN2B subunits in microglia-depleted mice, as GluN2A-dominant NMDA receptors have faster decay times (Chen et al., 1999). Taken together, these data indicate that microglia play a role in regulating the level of several synaptic proteins that are important for the function of glutamatergic excitatory synapses in the brain.

Removal of BDNF from Microglia Reduces Learning-Dependent Synaptic Structural Plasticity

The above results show that microglia deletion causes a reduction in several synaptic proteins involved in synaptic plasticity and function. However, the molecular mechanisms underlying the interaction between microglia and neurons remain unknown. To address this question, we investigated the role of brain-derived neurotrophic factor (BDNF) in mediating microglial-neuron interactions. We choose to study microglial BDNF for two reasons: First, BDNF exists in many cell types, including microglia, and BDNF from microglia has been shown to modulate neuronal plasticity in a mouse model of neuropathic pain (Coull et al., 2005). Second, BDNF is a potent regulator of synaptic development and plasticity (Chao, 2003), and increases dendritic spine plasticity in the adult cortex (Chakravarthy et al., 2006), GluN2B activation (Levine et al., 1998), and the level of VGlut1 (Melo et al., 2013). Given our findings that microglia depletion causes a reduction of GluN2B and VGlut1 as well as dendritic spine plasticity, it is possible that a lack of microglial BDNF may underlie some of the phenotypes associated with microglia depletion. To test this possibility, we crossed *CX_3CR1^{CreER}* mice with mice containing a floxed allele of *BDNF* (*$BDNF^{flox}$*) (Rios et al., 2001) to remove BDNF from microglia. We first validated this approach by sorting CX_3CR1-EYFP$^-$ cells or CX_3CR1-EYFP$^+$ microglia from the brains of *$CX_3CR1^{CreER/+}$:$BDNF^{fl/+}$* (*$BDNF^{fl/+}$*), or *$CX_3CR1^{CreER/+}$:$BDNF^{fl/fl}$*

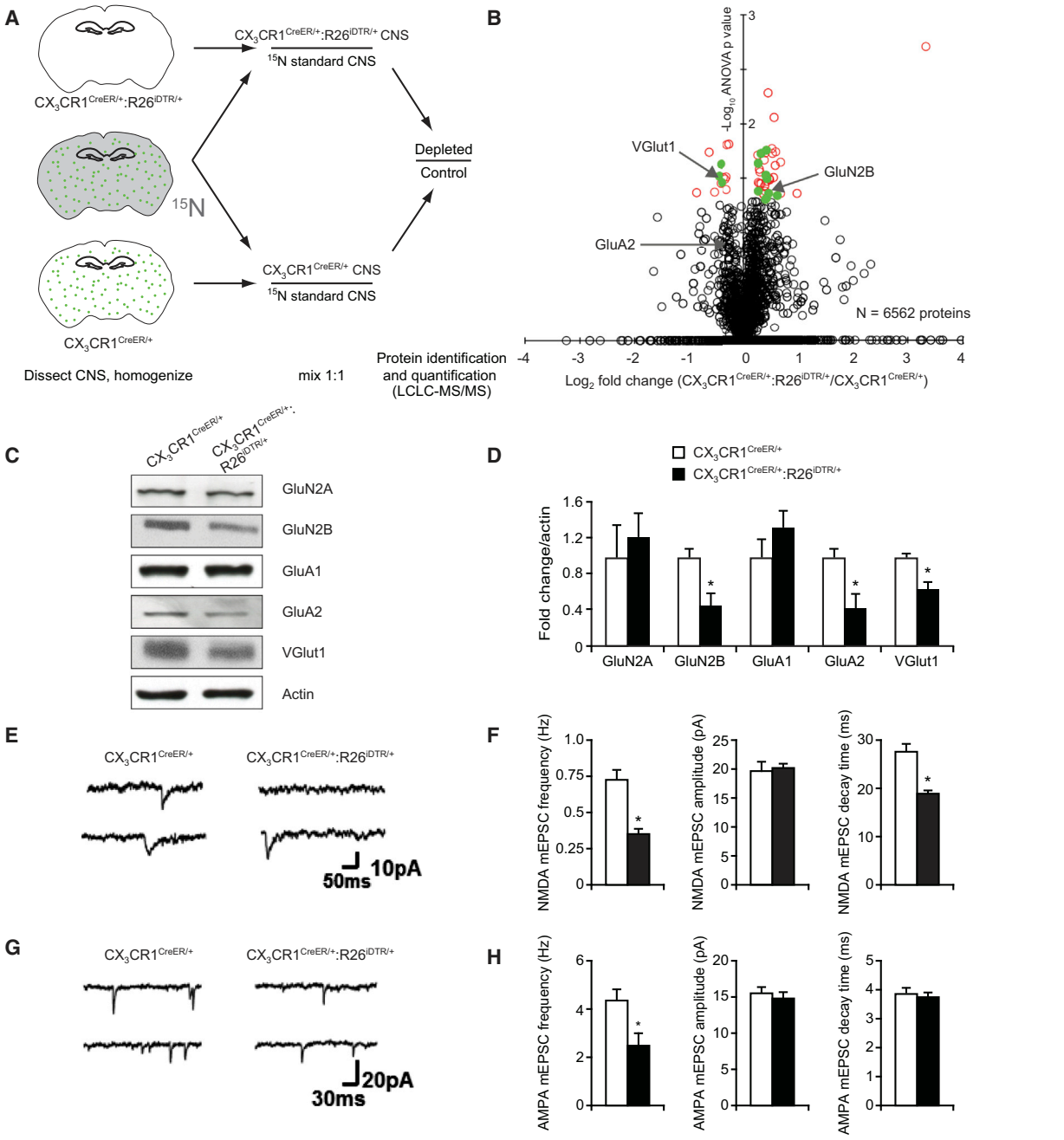

Figure 4. Biochemical and Electrophysiological Properties of Synapses Are Altered in Microglia-Depleted Brains

(A) Quantitative proteomic scheme to identify CNS proteins altered after microglial depletion. Control (n = 3) or microglia-depleted (n = 3) brain homogenates were mixed 1:1 with ^{15}N internal standard and prepared together. Samples were then analyzed by LCLC-MS/MS shotgun proteomics. Green dots represent microglia.

(B) Proteomic summary volcano plot (x axis = $\log_2$ *$CX_3CR1^{CreER/+}/CX_3CR1^{CreER/+}:R26^{iDTR/+}$*; y axis = $-\log_{10}$ ANOVA p value). Black open circles: quantified proteins; red open circles: significantly altered proteins; green filled circles: significantly altered proteins with known synaptic functions.

(C) Synaptosome fractions from control or microglia-depleted brains probed with indicated antibodies by western blot.

(D) Densitometric quantification of western blots in (C) (*$p < 0.05$, n = 6).

(E) Examples of NMDA mEPSCs in layer V pyramidal neurons from control and microglia-depleted mice.

(F) Average NMDA mEPSC frequency, amplitude, and decay time in control (n = 17 cells) and microglia-depleted mice (n = 17 cells). mEPSC frequency and decay time were significantly reduced in microglia-depleted mice ($p < 0.001$).

(G) Examples of AMPA mEPSCs in layer V pyramidal neurons from control and microglia-depleted mice.

(H) Average mEPSC frequency, amplitude, and decay time in control (n = 9 cells) and microglia-depleted mice (n = 8 cells). mEPSC frequency was significantly reduced in microglia-depleted mice ($p < 0.05$). Data are represented as mean ± SEM.

See also Table S1.

(*BDNF*$^{fl/fl}$) mice that had been given tamoxifen (Figure S5A) and assayed for recombination of the conditional *BDNF* allele using a PCR-based strategy. As expected, no recombination was observed in CX_3CR1-EYFP$^-$ cells. In contrast, robust recombination of the floxed allele was observed in CX_3CR1-EYFP$^+$ microglia from *BDNF*$^{fl/+}$ or *BDNF*$^{fl/fl}$ mice (Figure 5A). After administration of tamoxifen, the level of *BDNF* mRNA in microglia from *CX$_3$CR1*$^{CreER/+}$*:BDNF*$^{fl/fl}$ mice was markedly reduced as compared with *CX$_3$CR1*$^{CreER/+}$*:BDNF*$^{fl/+}$ controls (Figure 5B). Interestingly, the overall levels of BDNF protein in the cortex and hippocampus of *CX$_3$CR1*$^{CreER/+}$*:BDNF*$^{fl/fl}$ mice were unchanged compared with *CX$_3$CR1*$^{CreER/+}$*:BDNF*$^{fl/+}$ controls (Figure 5C). Taken together, these results indicate that BDNF can be specifically removed from microglia without causing a significant alteration in the total level of BDNF protein in the cortex or hippocampus.

To investigate the potential impact of microglial BDNF removal, we first stained the motor cortex and hippocampal CA1 region for NeuN, cleaved caspase 3, or SV2 but found no significant difference in these parameters between microglial BDNF-deleted and control mice (Figures S5B–S5D). Thus, microglial BDNF removal does not alter the overall densities of neurons or synapses in the cortex or hippocampus. We next asked whether removal of BDNF from microglia had any effects on the level of synaptic protein expression, motor-learning-induced synaptic plasticity, and performance improvement after learning. We performed western blot analysis of synaptosomes and found a significant decrease in the levels of GluN2B and VGlut1, but not GluA2, in mice lacking microglial BDNF (*BDNF*$^{fl/fl}$) as compared with control mice (*BDNF*$^{fl/+}$) (Figures 5D and 5E). Importantly, BDNF removal from microglia resulted in a significant decrease in motor-learning-induced spine formation, but not spine elimination, over 2 days (Figures 5F and 5G). Furthermore, similar to what was observed for microglia-depleted animals, whereas BDNF removal from microglia had no effect on baseline rotarod performance, mice lacking microglial BDNF demonstrated a reduction in performance improvement after motor training as compared to controls (Figures 5H and 5I). Mice lacking microglial BDNF also showed significantly reduced freezing fear response to the conditioned auditory cue stimulus during the recall test (Figure 5J). In the NOR task, however, mice lacking microglial BDNF did not show a significant difference from the control mice (Figure 5K). Taken together, these results demonstrate that loss of microglial BDNF recapitulates several defects observed in microglia-depleted mice and suggest that microglial BDNF is an important regulator of learning-induced synaptic formation and behavioral performance.

Microglial BDNF Affects Synaptic Plasticity via the Tropomyosin-Related Kinase Receptor B Signaling Pathway

As microglial processes are located in close proximity to synapses, microglial BDNF could be released to directly affect the structure and function of nearby synapses. Alternatively, microglial BDNF could have a role in microglial differentiation or homeostasis, and the removal of BDNF from microglia might act in an autocrine fashion and indirectly affect synaptic plasticity. To distinguish between these possibilities, we first compared the overall number, rate of proliferation, and tissue distribution of microglia between *CX$_3$CR1*$^{CreER/+}$*:BDNF*$^{fl/fl}$ and *CX$_3$CR1*$^{CreER/+}$*:BDNF*$^{fl/+}$ controls, but found no significant differences in any of these parameters (Figures S6A–S6E). To examine whether loss of microglial BDNF might alter the association of microglial processes with dendritic spines, we measured the percentage of spines of layer V pyramidal neurons in the motor cortex that had Iba1$^+$ microglial processes within 1 μm and found no significant difference between *CX$_3$CR1*$^{CreER/+}$*:BDNF*$^{fl/fl}$ *and CX$_3$CR1*$^{CreER/+}$*:BDNF*$^{fl/+}$ control mice (Figures S6F and S6G). These results demonstrate that loss of BDNF from microglia does not alter the number, proliferation, or distribution of microglia, or the spatial association of microglial processes with dendritic spines.

The above findings suggest that microglial BDNF may be released to directly affect the plasticity of nearby synapses. BDNF is produced within neurons as a propeptide (proBDNF) that is subsequently cleaved to generate mature BDNF (mBDNF). Because the pro and mature forms of BDNF bind different receptors on the surface of neurons (p75/sortilin and tropomyosin-related kinase receptor B [TrkB], respectively) to affect different cellular processes (Chao, 2003), we asked whether, and in which form, BDNF might be produced and secreted by microglia. Because endogenous BDNF protein is difficult to detect due to low abundance (Herzog et al., 1994), we utilized BDNF-hemagglutinin knockin mice (*BDNF-HA*) in which the *BDNF* gene is replaced by HA-tagged BDNF (Yang et al., 2009b). We generated purified cultures of hippocampal neurons or microglia from P1 *BDNF-HA/HA* mice (microglial purity 98.6% ± 0.1%; Figure S6H) and immunoprecipitated HA-tagged BDNF from either the cell lysates or culture media of both cell types. In agreement with previous results, significant quantities of both proBDNF and mBDNF could be detected in both cell lysates and culture media from BDNF-HA neurons. Notably, both forms of BDNF were also detected in cell lysates and culture media from pure microglia cultures although the distribution of pro-BDNF to mBDNF in microglial lysates appeared to be weighted toward the mature form (Figure 6A). These results demonstrate that microglia are capable of producing and secreting both pro-BDNF and mBDNF.

It is well established that mature BDNF binds to TrkB receptors located on neurons, and binding of mBDNF to TrkB triggers a signaling cascade that promotes autophosphorylation of TrkB and synaptic plasticity (Chao, 2003). Consistent with an interaction between microglia BDNF and TrkB receptors, western blot analysis of synaptosomes showed a significant decrease in the levels of phosphorylated TrkB (p-TrkB) in *CX$_3$CR1* $^{CreER/+}$*:BDNF*$^{fl/fl}$ mice lacking microglial BDNF as compared with control *CX$_3$CR1* $^{CreER/+}$*:BDNF*$^{fl/+}$ mice (Figure 6B). To investigate microglia BDNF and TrkB signaling further, we tested the ability of the media from cultures of purified microglia (microglia-conditioned media [MCM]) from *CX$_3$CR1*$^{CreER/+}$*:BDNF*$^{+/+}$ or *CX$_3$CR1*$^{CreER/+}$*:BDNF*$^{flox/flox}$ P1 mice to phosphorylate TrkB in cultures of purified cortical neurons. Within 30 min, MCM from *CX$_3$CR1*$^{CreER/+}$*:BDNF*$^{+/+}$ mice was able to significantly increase the phosphorylation of TrkB compared with baseline levels in untreated neuronal cultures. Importantly, MCM generated from microglial

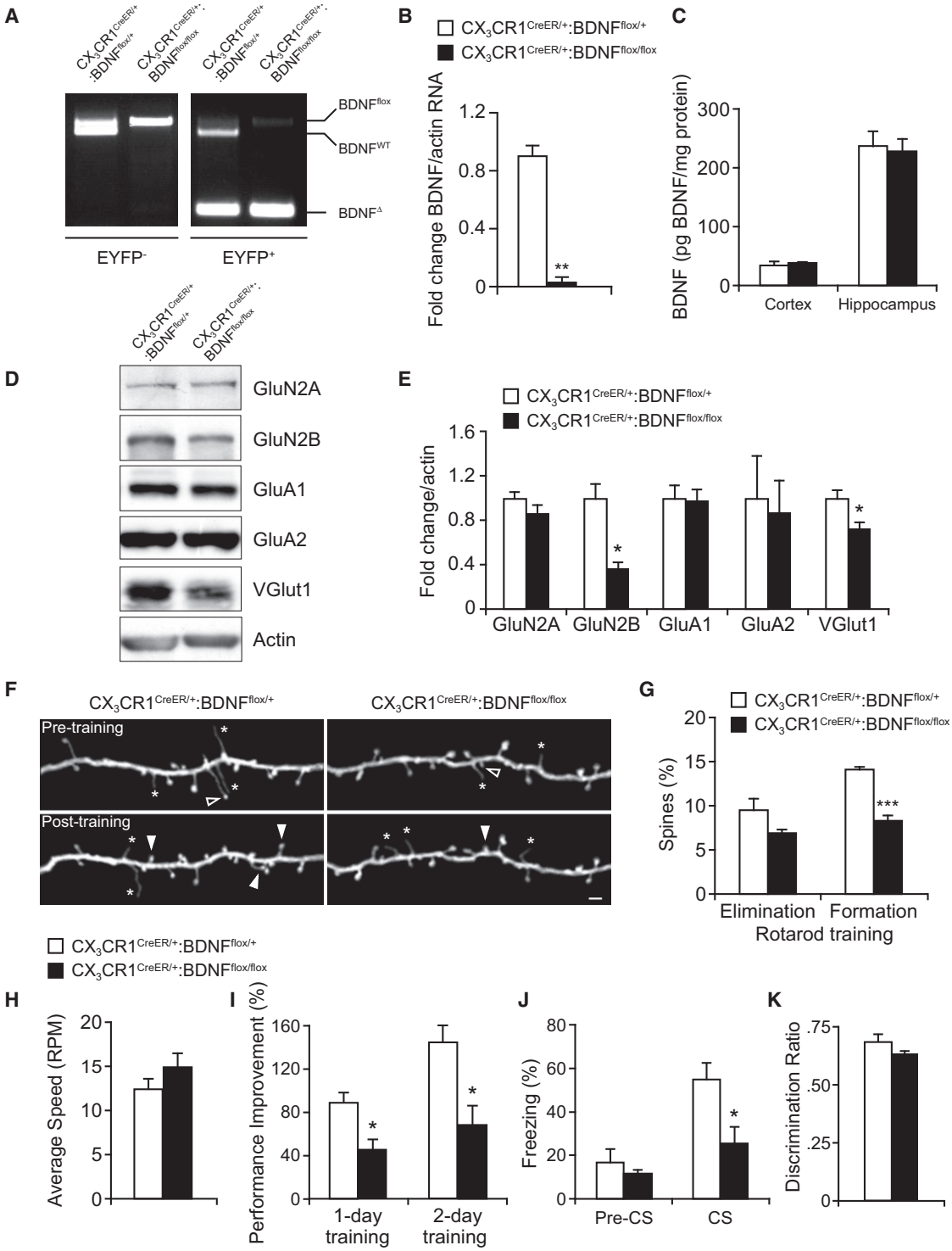

Figure 5. Loss of Microglial BDNF Results in Altered Synaptic Protein Levels, Synaptic Structural Plasticity, and Performance Improvement after Learning

(A) PCR-based analysis of WT ($BDNF^{WT}$), conditional undeleted ($BDNF^{flox}$), and conditional deleted ($BDNF^{\Delta}$) BDNF alleles from CX_3CR1-$EYFP^{-}$ and CX_3CR1-$EYFP^{+}$ cells sorted from the CNS of $CX_3CR1^{CreER/+}$:$BDNF^{flox/+}$ or $CX_3CR1^{CreER/+}$:$BDNF^{flox/flox}$ after tamoxifen treatment.

(B) Quantitative real-time PCR analysis of *BDNF* mRNA isolated from CX_3CR1-$EYFP^{+}$ microglia purified from $BDNF^{flox/+}$ or $BDNF^{flox/flox}$ mice (**$p < 0.01$, $n = 3$).

(C) Average protein levels of total BDNF in the cortex or hippocampus of $CX_3CR1^{CreER/+}$:$BDNF^{flox/+}$ or $CX_3CR1^{CreER/+}$:$BDNF^{flox/flox}$ mice as measured by ELISA ($n = 4$).

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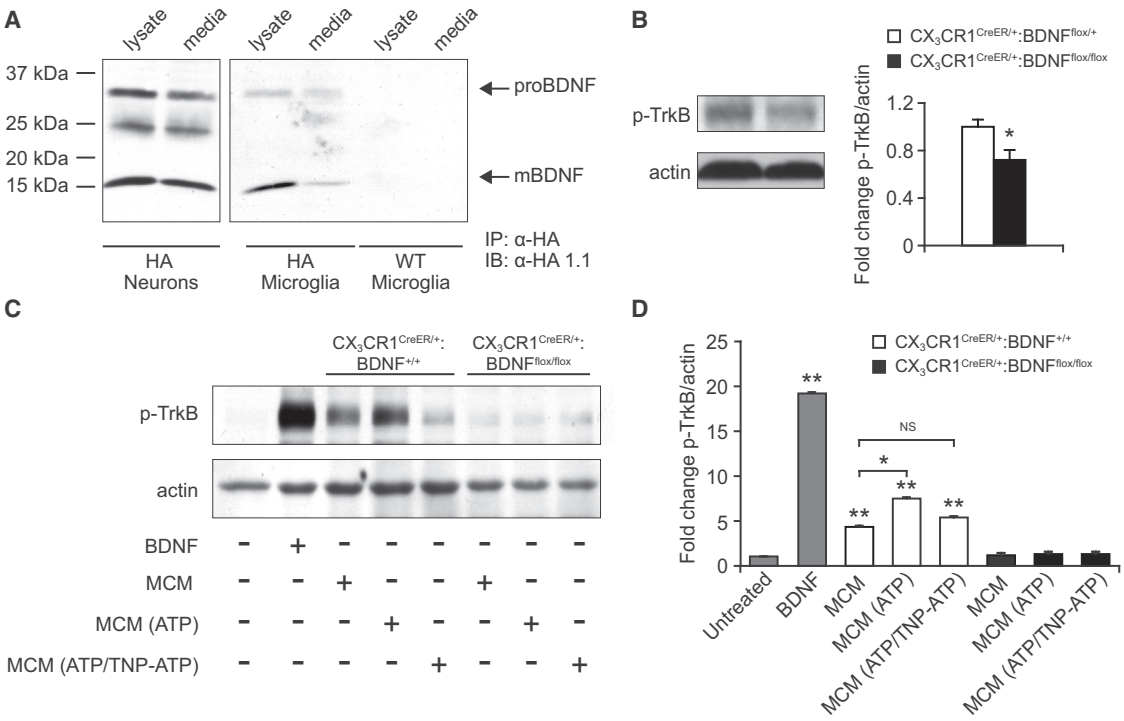

Figure 6. Microglia Produce Both Pro-BDNF and mBDNF to Phosphorylate Neuronal TrkB

(A) Neurons or microglia were cultured from P1 mice from BDNF-HA or WT animals. Cell lysates or culture media were immunoprecipitated with a rabbit antibody to HA. Pro-BDNF and mBDNF were detected by immunoblotting with a second antibody to HA (mouse HA1.1).

(B) Synaptosome westerns for p-TrkB from *$CX_3CR1^{CreER/+}$:$BDNF^{flox/+}$* or *$CX_3CR1^{CreER/+}$:$BDNF^{flox/flox}$* mice (*$p < 0.05$, n = 6).

(C) Representative immunoblots of E18 rat neurons at 8 days in vitro, treated as indicated.

(D) Densitometric quantification of p-TrkB western blots in (C) (*$p < 0.05$, **$p < 0.005$, n = 9). Data are represented as mean ± SEM.

See also Figure S6.

cultures devoid of BDNF (Figure S6I) failed to increase the phosphorylation of TrkB (Figures 6C and 6D). Previous studies demonstrated that ATP acting through the purinergic receptor $P2X_4$ is able to increase both the synthesis and release of BDNF from microglia in vitro (Trang et al., 2009). To test whether ATP and/or $P2X_4R$ are also involved in the ability of MCM to phosphorylate TrkB, we treated purified cultures of microglia with ATP or with ATP and the $P2X_4R$ antagonist 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) before harvesting MCM. Pretreatment of microglia cultures with ATP significantly increased the ability of MCM to phosphorylate TrkB, and this increase was blocked by the addition of TNP-ATP to the cultures (Figures 6C and 6D). Taken together, these findings suggest that microglial BDNF promotes synaptic structural plasticity involving activation of TrkB signaling.

DISCUSSION

The lack of tools to specifically perturb microglia at defined time points in a living animal has made it difficult to delineate the

(D) Synaptosome fractions from the brains of *$CX_3CR1^{CreER/+}$:$BDNF^{flox/+}$* or *$CX_3CR1^{CreER/+}$:$BDNF^{flox/flox}$* mice probed with indicated antibodies.

(E) Densitometric quantification of western blots in (D) (*$p < 0.05$, n = 6).

(F) Transcranial two-photon imaging of dendritic spines in Thy1 YFP mice crossed with *$CX_3CR1^{CreER/+}$:$BDNF^{flox/+}$* or *$CX_3CR1^{CreER/+}$:$BDNF^{flox/flox}$* mice before or after rotarod training. Filled and empty arrowheads indicate spines that were formed or eliminated, respectively, between two views. Asterisk indicates filopodia. Scale bar, 2 μm.

(G) Percentage of existing spines that were eliminated or new spines that formed over 2 days of training in the motor cortex of *$BDNF^{flox/+}$* or *$BDNF^{flox/flox}$* mice (***$p < 0.001$, n = 4).

(H) Average speed reached during the first rotarod training session (n = 5–7).

(I) Performance increase in motor-learning task over 1 or 2 days of rotarod training (*$p < 0.05$; error bars, SEM; n = 5–7).

(J) Percentage of freezing in control *$CX_3CR1^{CreER/+}$:$BDNF^{flox/+}$* and *$CX_3CR1^{CreER/+}$:$BDNF^{flox/flox}$* mice before (pre-CS) and during (CS) presentation of the conditioned stimulus in the recall test (*$p < 0.05$, n = 6–7).

(K) The discrimination ratio of time spent interacting with a novel object versus a familiar object in a NOR assay was significantly altered in mice depleted of microglial BDNF (*$p < 0.05$, n = 8). Data are represented as mean ± SEM.

See also Figure S5.

precise functions of microglia in the physiological brain. In this work, we have generated a tool that allows the specific and inducible manipulation of microglial numbers and functions within the CNS. Using this tool, we have revealed a physiological role of microglia in learning and learning-associated synaptic structural remodeling. Moreover, the use of CX_3CR1^{CreER} mice allowed us to uncover an important role of microglial BDNF in these processes.

Interactions between Microglia and Neurons: Promoting Learning-Induced Synapse Formation

Microglial processes are constantly motile in the physiological brain and are found in close proximity to synapses in both the early postnatal and adult cortex (Davalos et al., 2005; Tremblay et al., 2010; Wake et al., 2009). The functional outcome of microglial interaction with neurons appears to depend on the developmental stage of the CNS. In the embryonic brain, which has high levels of programmed neuronal cell death, microglia have been observed to phagocytose apoptotic neurons (Peri and Nüsslein-Volhard, 2008) or actively participate in the killing of neurons (Marín-Teva et al., 2004). In the embryonic zebrafish optic tectum, microglia appear to play a role in the homeostasis of neuronal activity by silencing neurons through a contact-dependent mechanism (Li et al., 2012). Recent work has shown that mice lacking *CX_3CR1* have delayed invasion of the cortical barrel fields by microglia, and a transient delay in the normally observed AMPA receptor subunit switching in thalamocortical barrel cortex synapses (Hoshiko et al., 2012). Furthermore, it was recently proposed that microglia are involved in the active engulfment of synapses in the hippocampus and the dorsal lateral geniculate nucleus (dLGN) during early postnatal development (Paolicelli et al., 2011; Schafer et al., 2012). Pruning of synapses within the dLGN is thought to depend upon the presence of *CR3*, which may interact with synaptically localized C3 protein that acts as a positive signal for phagocytosis of presynaptic axonal terminals.

By taking advantage of CX_3CR1^{CreER} mice and transcranial two-photon imaging, we observed a significant decrease in learning-induced formation of postsynaptic dendritic spines in the absence of microglia or microglial BDNF in young and mature adult mice. Two lines of evidence suggest that the main synaptic function of microglia in the mature brain is the promotion of dendritic spine formation rather than spine elimination. First, removal of BDNF from microglia causes a significant decrease in learning-induced spine formation, but not spine elimination, over 2 days. Second, previous studies have shown that motor-learning-induced spine formation occurs 2 days before spine elimination, and that the degrees of spine formation and elimination are strongly correlated (Liston et al., 2013; Yang et al., 2009a). Therefore, the decreased spine elimination observed in the absence of microglia may simply be a result of fewer spines being formed after motor learning. It is worth noting that we also observed a decrease in spine formation and elimination at P19. This raises the possibility that microglia have an important function in promoting synapse formation not only during the late postnatal stage and adulthood but also during the early postnatal period (e.g., the first postnatal week), when both synaptogenesis and pruning are extensive.

Previous studies have shown that learning-dependent synapse formation strongly correlates with performance improvement after learning (Liston et al., 2013; Yang et al., 2009a). Concomitantly with the reduction of learning-induced spine formation, we observed a significant decrease in performance improvement after rotarod learning in the absence of microglia or microglial BDNF. Additionally, we found that microglia have important roles in fear learning and NOR tasks. These results underscore the important function of microglia in forming structural correlates of learning experiences that are critical for behavioral performance. These findings also suggest that microglial dysfunctions may cause a reduction of learning-dependent synaptic formation and contribute to learning deficits in neurological diseases. Furthermore, because activation of microglia is common in many neurodegenerative diseases and accompanies alterations of gene expression, microglia activation may compromise the physiological function of microglia and contribute to the disease pathophysiology. Future use of CX_3CR1^{CreER} mice to activate and inactivate important signaling pathways in microglia will aid in addressing these questions.

The Role of Microglial BDNF in Synaptic Plasticity and Function

The neurotrophin BDNF is a critical mediator of neuronal survival, differentiation, and plasticity (Chao, 2003). Although the major source of BDNF in the adult brain appears to be neurons (Rauskolb et al., 2010), BDNF can also be detected in oligodendrocytes, astrocytes, and microglia (Dougherty et al., 2000). Through conditional gene inactivation, we have determined that microglial BDNF also plays an important role in the healthy brain by regulating learning-induced synapse formation. Our results showing that the overall levels of BDNF protein do not change in the absence of microglial BDNF are in agreement with previous findings that small changes in BDNF levels or the presence of a SNP in the *BDNF* gene could have significant neurological effects (Greenberg et al., 2009). Because microglial processes are highly motile and located in close proximity to synaptic terminals (Davalos et al., 2005; Tremblay et al., 2010; Wake et al., 2009), our data raise the possibility that the release of BDNF from microglia may be regulated in a localized and/or activity-dependent fashion. Indeed, the majority of BDNF release by neurons in the adult brain appears to be triggered by neuronal activity (Balkowiec and Katz, 2000). It is attractive to speculate that BDNF may be secreted from microglial processes to specifically modulate a subset of synaptic connections involved in a particular learning task.

Through what mechanisms does microglial BDNF exert its effects on the CNS? Our results suggest that similar to neuronal BDNF, microglial BDNF could act on neuronal TrkB and modulate glutamatergic synaptic transmission and plasticity (Rex et al., 2007). Microglial BDNF could also affect inhibitory synaptic transmission via TrkB signaling, as has been shown in the spinal cord (Coull et al., 2005) and hippocampus (Zheng et al., 2011). In addition, the microglia themselves express activated TrkB, and BDNF released from microglia has been shown to increase their proliferation (Spencer-Segal et al., 2011). Although we are unable to detect changes in the number or spatial distribution of microglia in mice lacking microglial BDNF, it would be

interesting to investigate whether microglial BDNF may act in an autocrine manner to influence microglial function. An equally interesting area for further investigation is the signals that trigger BDNF release from microglia within the cortex. Several lines of evidence suggest that a major stimulus for BDNF release from microglia is the binding of ATP to the purinergic receptor $P2X_4R$. Because ATP is released at sites of active synaptic transmission (Khakh and North, 2012) and acts as a robust chemoattractant for microglial processes (Davalos et al., 2005), one intriguing possibility is that ATP recruits microglial processes to active synapses where they subsequently release BDNF. Our in vitro experiments suggest that $P2X_4$ receptors could play an important role in promoting BDNF release and TrkB phosphorylation. However, it remains to be determined whether the basal level of $P2X_4$ receptors is sufficient to promote BDNF release in the normal CNS, or whether other factors also contribute. Lastly, microglia may modulate synaptic functions in the healthy CNS that are independent of BDNF. Our proteomics screen identified numerous proteins with known synaptic functions that were altered in the absence of microglia (Table S1). Future studies using CX_3CR1^{CreER} mice are required to better understand how microglia impact synaptic plasticity and function via BDNF and other signaling pathways in both the developing and adult brain.

EXPERIMENTAL PROCEDURES

Mice

All mice were maintained in the Skirball Institute specific pathogen-free animal facility and handled in accordance with the institutional guidelines for animal care and use. Detailed information on the various mouse strains used is provided in the Extended Experimental Procedures.

Isolation of Tissues and Flow Cytometry

Single-cell suspensions were generated from harvested organs, stained, and analyzed by polychromatic flow cytometry.

Histology, Quantitative RT-PCR, and ELISA

These standard experiments were performed with commercially available kits and reagents. See the Extended Experimental Procedures for more information.

Mass Spectrometry

Mass spectrometry sample processing and analysis was performed as previously reported (Butko et al., 2013), except that the samples were analyzed on a LTQ-Orbitrap XL with 9 MS/MS per MS. The false discovery rate at the protein identification level was $\leq$1% for each analysis, based on a target-decoy strategy.

Isolation of Synaptosome Fractions and Western Blot

Mice were deeply anesthetized and perfused with 40 ml of Ca^{2+}/Mg^{2+}-free Dulbecco's PBS. Brain tissue was removed and synaptosomes were isolated as previously described (Gray and Whittaker, 1962).

Electrophysiology

Layer V pyramidal neurons were recorded in acute slices as previously described (Pattwell et al., 2012). All electrophysiological recording experiments were done in double-blind fashion.

In Vivo Transcranial Two-Photon Imaging

Dendritic spines in the mouse motor cortex were imaged with a two-photon microscope through a thinned-skull window. This procedure has been described in detail previously (Yang et al., 2009a). All of the imaging experiments and data analysis were performed in double-blind fashion.

Behavioral Testing Protocols

Rotarod training, cue-based FC, and NOR behavioral testing were performed as previously described (Bevins and Besheer, 2006; Lai et al., 2012; Yang et al., 2009a). All behavioral experiments were performed in double-blind fashion.

Detailed information on the experimental procedures used can be found in the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.11.030.

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